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A comparison between placental and amniotic mesenchymal stem cells for transamniotic stem cell therapy (TRASCET) in experimental spina bifida

Christina Feng, Christopher D. Graham, John Patrick Connors, Joseph Brazzo III, David Zurakowski, Dario O. Fauza *

Department of Surgery, Boston Children's Hospital and Harvard Medical School, Boston, MA

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ABSTRACT

Purpose: We compared placental-derived and amniotic fluid-derived mesenchymal stem cells (pMSCs and afMSCs, respectively) in transamniotic stem cell therapy (TRASCET) for experimental spina bifida.
Methods: Pregnant dams (n = 29) exposed to retinoic acid for the induction of fetal spina bifida were divided into four groups. Three groups received volume-matched intraamniotic injections of either saline (n = 38 fetuses) or a suspension of 2×10^6 cells/mL of syngeneic, labeled afMSCs (n = 73) or pMSCs (n = 115) on gestational day 17 (term = 21–22 days). Untreated fetuses served as controls. Animals were killed before term. Statistical comparisons were by Fisher's exact test ($p < 0.05$).
Results: Survival was similar across treatment groups ($p = 0.08$). In fetuses with isolated spina bifida (n = 100), there were higher percentages of defect coverage (either partial or complete) in both afMSC and pMSC groups compared with saline and untreated groups ($p < 0.001$–0.03 in pairwise comparisons). There were no differences in coverage rates between afMSC and pMSC groups ($p=0.94$) or between saline and untreated groups ($p=0.98$).
Conclusions: Both pMSC and afMSC can induce comparable rates of coverage of experimental spina bifida after concentrated intraamniotic injection in the rodent model. This broadens the options for timing and cell source for TRASCET as a potential alternative in the prenatal management of spina bifida.

Transamniotic stem cell therapy (TRASCET) has emerged experimentally as a potential alternative, or adjuvant, in the prenatal management of spina bifida. It is based on augmenting the biological role of mesenchymal stem cells (MSCs) that are naturally occurring in the amniotic fluid, which have been shown to enhance normal fetal wound healing [1]. Amniotic fluid-derived MSCs (afMSCs) injected in large numbers in the amniotic cavity have been shown to induce the formation of a rudimentary skin overlying the spina bifida defect, as well as to minimize the associated Chiari II malformation in a rodent model [2,3].

In addition to the amniotic fluid, multiple mesenchymal stem cell sources could be potential options for TRASCET, for which the optimal cell type still remains to be determined. Placenta-derived MSCs (pMSCs) are a clearly feasible alternative, as they can be relatively easily procured during pregnancy, for example through chorionic villus sampling (CVS) [4,5]. In this study, we aimed at comparing the effects of pMSCs and afMSCs used as agents of TRASCET in a rodent model of spina bifida.

1. Methods

This study was approved by the Boston Children's Hospital Institutional Animal Care and Use Committee under protocol # 15-07-2951R.

1.1. Donor cell procurement and processing

Normal isogenic Lewis rat dams served purely as afMSC donors on gestational day 21 (E21; term = 21–22 days). A midline laparotomy was performed and the bicornuate uterus eviscerated. Amniotic fluid from viable fetuses was obtained using a 30G needle (Becton Dickinson, Franklin Lakes, NJ) on a 1 mL syringe (Becton Dickinson) introduced into each amniotic cavity upon the ventral aspect of the fetus, through which amniotic fluid was procured. Syngeneic afMSCs were then isolated and expanded based on methods as we have previously described [5,6].

Donor pMSCs were also procured from normal isogenic Lewis rat dams that served purely as donors on E21. Syngeneic pMSCs were isolated and expanded based on our previously described methods [4,5]. For the isolation, in brief, a small amount of placental sample from which the maternal decidua was mechanically removed was minced in a sterile 10 cm tissue culture plate. A solution containing 0.1% (*w*/*v*) type II collagenase (Worthington Biochemical, Grand Rapids, MI) and

* Corresponding author at: Boston Children's Hospital, Department of Surgery, 300 Longwood Avenue - Fegan 3, Boston, MA, 02115. Tel.: +1 617 919 2966; fax: +1 617 730 0910.
E-mail address: dario.fauza@childrens.harvard.edu (D.O. Fauza).

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4.0 U dispase II/2.5 mM $CaCl_2$ per liter (Roche, Indianapolis, IN and Sigma-Aldrich, St. Louis, MO, respectively) was used to cover the tissue, mixed, and filtered through an 100 μm mesh (Fischer Scientific, Pittsburgh, PA) into a sterile 15 mL conical centrifuge tube. The specimen was centrifuged (2000 rpm × 3 min at room temperature), the pellet resuspended in 6 mL of mesenchymal-20 media (M-20), consisting of 400 mL Dulbecco's Modified Eagle Medium (DMEM, Corning, Corning, NY) + 100 mL Fetal Bovine Serum (Gibco, Carlsbad, CA) + 5 mL of antibiotic/antimycotic solution consisting of 10,000 IU/mL penicillin G, 10,000μg/mL streptomycn sulfate, and 250 ng/mL amphotericin B (all from Corning) + 5 ng/mL of recombinant human fibroblast growth factor (rhFGF) (Promega, Madison WI). One milliliter of the mixture was plated into a 6-well collagen coated plate (Life Technologies) for initial MSC isolation.

Fluorescence-activated cell sorting analysis using the Vantage SE cell sorter (Becton Dickinson Biosciences, East Rutherford NJ) was performed to confirm their mesenchymal progenitor identity with primary conjugated mouse monoclonal antibodies previously validated for use in rats, namely for CD29 (Becton Dickinson Biosciences); CD44 (R&D Systems, Minneapolis MN); CD45 (Invitrogen, Grand Island NY); and CD90 (Becton Dickinson Biosciences). A purified CD73 (Becton Dickinson Biosciences) conjugated with an anti-mouse IgG1 against purified CD73 (Biolegend, San Diego, CA) was also used. Cells were labeled by fluorescent nanocrystal technology using the Qtracker® cell labeling kit 525 (Life Technologies, Chicago, IL) emitting a wavelength of 525 nm with excitation at 405–485 nm, as per the manufacturer's instructions [7]. Green fluorescence was observed in 80%–90% of cells postincubation using an EVOS® FL Color Imaging System microscope fitted with an on-board computer and integrated imaging software (Life Technologies). A small subset of afMSC cells was labeled with green fluorescent protein (GFP) using plasmid pMIG #9044 (Addgene, Cambridge MA) and Lipofectamine LTX with PLUS Reagent (Invitrogen). Cells were transfected in antibiotic-free mesenchymal media [450 mL DMEM (Fisher Scientific) + 50 mL FBS (Life Technologies)] for 6 h at 37 °C, and then refed with fresh M-20 media during expansion thereafter. GFP positivity was confirmed using the EVOS® FL Color Imaging System microscope.

1.2. Congenital spina bifida creation and intraamniotic injections

Twenty-nine time-dated pregnant Sprague–Dawley dams (Charles River, Wilmington, MA) were exposed to retinoic acid for the induction of fetal neural tube defects, as previously described [8]. A mixture of all-trans retinoic acid (Sigma-Aldrich Chemical, St. Louis, MO) was dissolved in olive oil to a concentration of 10 mg/mL at room temperature. After exposure to isoflurane (Abbot Laboratories, North Chicago, IL), chamber inhaled at 2%–4% in 100% oxygen, the dams received, through gavage, 60 mg/kg of the all-trans retinoic acid solution on E10, between 6:00 pm and 8:00 pm.

All animals were then divided into four groups. The untreated group had no further manipulation. The other three groups received volume-matched intraamniotic injection blindly to all fetuses of either saline ($n = 38$ fetuses), or a suspension of 2×10^6 cells/mL of afMSCs ($n = 73$), or of pMSCs ($n = 115$) on E17.

Intraamniotic injections were performed according to previously developed methods [2]. In brief, general anesthesia was induced and maintained with isoflurane (Abbot), chamber inhaled at 2%–4% in 100% oxygen. A large midline incision was made and the uterus eviscerated. A 33G non-coring needle on a 100 μL syringe (Hamilton Company, Reno, NV) was used to inject the desired solution into each amniotic cavity containing a viable fetus by its ventral aspect, carefully avoiding it, the placenta, and the umbilical cord. The uterus was returned to the abdomen and the incision closed in two layers, using 3-0 Prolene (Ethicon, Somerville, NJ) and 5-0 Vicryl (Ethicon) running sutures. Animals were allowed to recover and postoperative analgesia with buprenorphine (Reckitt and Colman Pharmaceuticals, Richmond, VA) was used as needed.

1.3. Spina bifida analysis

All dams were euthanized with chamber-inhaled carbon dioxide just prior to term, on E21. Fetuses were procured and examined for the presence or absence of an isolated spina bifida. The presence or absence of any degree of coverage over the spina bifida defect was evaluated by gross inspection, but defined as present only if confirmed as rudimentary skin on histology by three blinded observers. For histology, specimens were paraffin-embedded and stained with hematoxylin–eosin (H&E). Coverage was determined to be partial or complete based on previously published methods [2,3].

Screening for fluorescent nanocrystal-labeled cells was performed in unstained slides using an EVOS® FL Color Imaging System microscope fitted with an on-board computer and integrated imaging software (Life Technologies).

1.4. Statistical analysis

Statistical analysis of fetal survival, percentage of fetuses with isolated spina bifida, and prenatal coverage of the defect was based on Fisher's exact test for comparing binary proportions with a two-tailed $p < 0.05$ as the criterion for statistical significance. An additional, complementary analysis based on logistic regression with the Wald test to assess differences between treatment groups was also performed since it is less conservative than Fisher's exact test and has increased statistical power for detecting differences between treatment groups. Data analysis used IBM/SPSS Statistics (version 21.0, IBM, Armonk, NY).

2. Results

A total of 208 fetuses were viable at euthanasia. Of those, 119 fetuses (57%) had a neural tube defect (spina bifida with or without concomitant exencephaly) and 100 fetuses (48%) had an isolated spina bifida defect. Fetal survival was 89% for the afMSC group (65/73), 78% for the pMSC group (90/115), and 79% for the saline group (30/38). There were no significant differences in fetal survival between afMSC vs. pMSC ($p = 0.07$), afMSC vs. saline ($p = 0.15$), or pMSC vs. saline ($p = 0.93$). The presence of spina bifida was significantly lower in afMSC- (55%) and pMSC-treated groups (43%) compared to the saline group (83%) (both $p < 0.001$) and to the untreated group (both $p < 0.001$). No significant difference was observed in the percent of fetuses with spina bifida between afMSC and pMSC groups ($p = 0.15$) or between saline versus untreated groups (both 83%, $p = 1.00$).

Among surviving fetuses with isolated spina bifida, the percentage of fetuses with any degree of coverage of the defect (partial or complete) was 46% in the afMSC group (13/28), 47% in the pMSC group (18/38), 0% for saline (0/22), and 8% for the untreated group (1/12). Analysis by logistic regression and the Wald test confirmed significant differences in any coverage between afMSC and pMSC compared to saline (each $p < 0.001$), as well as between afMSC and untreated ($p = 0.042$), and between pMSC and untreated ($p = 0.036$). No significant differences in any degree of coverage were found between the afMSC and pMSC groups ($p = 0.94$), or between saline and untreated groups ($p = 0.98$) (Fig. 1).

The percentage of fetuses with partial coverage of the defect was 36% in the afMSC group (10/28), 40% in the pMSC group (16/38), 0% for the saline group (0/22), and 8% for the untreated group (1/12). Analysis by logistic regression confirmed significant differences between afMSC and pMSC compared to the saline group (each $p < 0.001$), and between pMSC and the untreated group ($p = 0.039$). No differences in partial coverage were found between afMSC and pMSC ($p = 0.60$), between afMSC and the untreated group ($p = 0.11$), or between saline and the untreated group ($p = 0.98$). Complete coverage of the defect was not